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SCREENING AND CHARACTERISATION OF ACC DEAMINASE PRODUCING BACTERIA FROM RHIZOSPHERE OF SUNFLOWER *HELIANTHUS ANNUUS* L.

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ABSTRACT

Due to the adverse effects of polluting chemicals and other stresses the growth of various crop plants is affected. The activity of soil microbes sometimes helps to combat such stress effects.One such activity is the production of ACC (1-Aminocyclopropane-1-Carboxylic acid) deaminase by microorganisms particularly under unfavourable environmental conditions such as flooding, heavy metals, phytopathogens, drought and high salt. Ethylene is an important phytohormone, but over-produced ethylene under stressful conditions can result in the inhibition of plant growth or death, especially for seedlings.This study was undertaken to isolate ACC deaminase producing bacteria from the rhizosphere and associated regions of an important oleaginous plantSunflower (Helianthusannuus L). Pseudomonas and Enterobacter species showed good growth when grown in DF salts minimal mediumandNFb medium amended with ACC as a sole source of Nitrogen. Root elongation was also observed in Pseudomonas and Enterobacter species. These bacteria were also promoting growth during early stages. Such bacteria could be used for plant growth promotion under stressful conditions and even in several bioremediation studies.

KEYWORDS: Phytopathogens; Ethylene; Phytohormone; ACC Deaminase

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Original Article

INTRODUCTION

Agricultural crops are exposed to many stresses that are induced by both biotic and abiotic factors. These stresses decrease yields of crops and represent barriers to the introduction of crop plants into areas that are not suitable for crop cultivation. The occurrence and activity of soil microorganisms are affected by a variety of environmental factors as well as plant-related factors (species, age). Abiotic stress factors include high and low temperature, salinity, drought, flooding, ultraviolet light, air pollution (ozone) and heavy metals. The yield losses associated with abiotic stresses can reach 50% to 82%, depending on the crop. In many semi-arid and arid regions of the world, crop yield is limited due to increasing salinity of irrigation water as well as soil salinity. Under high salinity, plants exhibit a reduced leaf growth rate due to decreased water uptake, which restricts photosynthetic capacity. Plant involves a number of metabolic and physiological changes in response to salt stress and drought (Hu, 2005). The inoculation of salt-stressed plants with PGPR strains alleviates the salinity stress in plants. Soil salinity is one of the most severe factors limiting nodulation, yield and physiological response in soybean. An increase in salinity in the soil causes a physiological response or disorder in lettuce plants (Han and Lee, 2005).

The PGPR containing ACC deaminase are present in various soils and offer promise as a bacterial

inoculum for improvement of plant growth, particularly under unfavourable environmental conditions such as flooding, heavy metals, phytopathogens, drought and high salt. Ethylene is an important phytohormone, but over-produced ethylene under stressful conditions can result in the inhibition of plant growth or death, especially for seedlings. PGPR containing ACC deaminase can hydrolyze ACC, the immediate precursor of ethylene, to α-ketobutarate and ammonia, and in this way promote plant growth. Inoculation of crops with ACC deaminase-containing PGPR may assist plant growth by alleviating deleterious effects of salt stress ethylene (Belimov et al., 2001). (Castango et al., 2008;Alikhani, Saleh-Rastin and Antoun, 2006;Seshadri et al., 2007). The metal resistant Plant growth promoting bacteria (PGPB) can serve as an effective metal sequestering and growth-promoting bioinoculant for plants in metal stressed soil (Rajkumar and Freitas, 2008). The deleterious effects of heavy metals taken up from the environment on plants can be lessening with the use of PGP bacteria or mycorrhizal fungi (Tam, 1995;Belimove et al., 2005; Denton, 2007). The soil microbes, plant growth promoting rhizobacteria (PGPR), P solubilizing bacteria, mycorrhizal-helping bacteria (MHB) and arbuscularmycorrhizal fungi (AMF) in the rhizosphere of plants growing on trace metal contaminated soils plays an important role in phytoremediation (Khan,2005). Phytoremediation provides a cheap, energy efficient detoxification method that manipulates intrinsic plant characteristics to concentrate the metal contamination in shoot biomass and reduce the bioavailability of the heavy metals. Soil microbes mitigate toxic effects of heavy metals on the plants through secretion of acids, proteins, phytoantibiotics, and other chemicals (Denton, 2007). A multi-process phytoremediation system (MPPS) utilizes plant/PGPR (plant growth promoting rhizobacteria) interactions to mitigate stress ethylene effects, thereby greatly increasing plant biomass, particularly in the rhizosphere and it also causes the decontamination of persistent petroleum and organic contaminants in soil (Greenberg et al., 2006).

Ethylene is a plant hormone that is involved in the regulation of many physiological responses (Zahir et al., 2008). Ethylene was originally regarded as a stress hormone because its synthesis in plants is increased by a number of biotic and abiotic stresses. Ethylene production has often been associated with reduced growth and premature senescence and may be an indicator of plant susceptibility to stresses such as drought and heat (Jacobson, Pasternak and Glick, 1994; Glick, Karaturovic and Ewell, 1995; Shah et al., 1998). The activity of these PGPR may be helpful as ACC deaminase containing PGPR markedly lowerthe level of ACC in the stressed plants thereby limiting the amount of stress ethylene synthesis and hence the damage to the plant. These bacteria are beneficial to plant growth since in the natural environment plants are often subjected to ethylene produced stresses. So this activity will be helpful in agriculture and horticultural settings as well as environmental clean up (Phytoremediation) protocols (Penrose and Glick, 2003). The present study was undertaken with the focus to isolate the ACC deaminase producing bacteria in the rhizosphere and associated regions of Sunflower plants in the Southern part of Gujarat,India and improving growth by inoculation with PGPR possessing several plant growth promoting mechanisms for sunflower one of the important oil crop plant in India and particularly in Gujarat.

MATERIALS AND METHODS

Samples were collected and studies were carried out during year 2009-2011 from sunflower field situated in Mankana village in Kamrej taluka, Dist. Surat in the southern part of Gujarat. In the present study the samples considered were Non-Rhizosphere soil (Bulk Soil) that detaches from the root when the plant is shaken, Rhizosphere soil (fraction of soil that remains attached to the root) and along with these, rhizoplane and endorhizosphere (endophytic) region samples associated with *Helianthus annuus* plant roots were also collected, processed and were then analyzed. (Wocoma, 2008).

Detection of ACC Deaminase Activity by Broth Assay Experiment

The rhizobacteria were screened based on the ability to utilize the compound 1-aminocyclopropane-1-carboxylic acid (ACC) as a sole nitrogen source using the procedure of Penrose and Glick. Bacterial isolates were aerobically cultured at room temperatures (21±1°C) in DF salts minimal medium with ACC as a sole nitrogen source (+ACC). Bacterial growth in liquid media was monitored by measuring the optical density (OD) of the cultures at 600 nm (Penrose and Glick, 2003).

ACC metabolism assay was carried out to characterize the rhizobacterial strains for their ability to use ACC as sole nitrogen source. The strains were grown on two nitrogen sources (ACC and Ammonium sulphate) and one mineral source (Magnesium sulphate), in a Minimal salts (MS) medium to observe the growth rate of the strain for ACC substrate in parallel to ammonium sulphate. The measurement of optical density was then carried out.(Zafar-ul-hye, 2007).

Plate Assay Experiment

ACC deaminase activity was determined by the method of Glick et al. (1995). For this, 1 µL of each LB pure bacterial culture was inoculated into agar plates containing NFb or NFb-ACC modified by addition of 1-aminocyclopropane-1-carboxylate (5.0 g l^{-1}) as a unique nitrogen source. Plates were incubated at 28°C and observed daily for colony formation for up to 4 days. Colonies were re-inoculated and incubated in the same experimental conditions. Newly formed colonies in NFb medium with addition of ACC were considered positive for ACC deaminase activity (Sgroy et al., 2009).

The cultures were also plated onto solid DF (Dworkin and Foster 1958) salts minimal medium and incubated for 48 hr at either 25 or 30°C. These plates were prepared with 1.8% bacto agar which had low nitrogen content and were spread with ACC just prior to use. Before streaking the ACC was allowed to dry fully. The inoculated plates were incubated at an appropriate temperature for 3 days and growth on the plates checked daily (Penrose and Glick, 2003).

BACTERIZATION EXPERIMENTS

Seed Germination (Plate Assay)

In all experiments, seeds of sunflower were wetted in 20 M ethanol for 5 seconds and surface sterilized in 0.21 M NaOCl for 5 min. The seeds were washed in sterile distilled water and soaked for 10 minutes in 0.01 M HCl (to remove traces of NaOCl) and washed 5 times in sterile distilled water to remove traces of HCl. All the bacterial isolates were grown on 0.1 x TSB for 24 to 48 hr, agitated on a rotary shaker and before harvest, centrifuged. The pellets were collected and suspensions prepared in 0.1 M $MgSO_4$ to give an absorbance of 0.1 at 620 nm (10^7-10^8 cells/ml). These suspensions were used for the bacterization. The sunflower seeds were bacterized with the selected test strains bysoaking 10 seeds in the above prepared bacterial suspensions for 30 minutes. Control seeds were treated with 0.1 M $MgSO_4$ only. After treatment the seeds were placed, 10 seeds per plate in 9 cm diameter glass petriplates lined with 2 filter papers, moistened with sterile de-ionized water (SDW) to test for germination. Petri plates were covered and incubated in the dark at ambient temperature. SDW was added to the plates to provide moisture for germination when necessary. Germination was recorded at every 24 hr (Wocoma, 2008; Egamberdiyeva, 2007).

Seed Bacterization (Pot Assay)

The method of Weller and Cook (1983) was followed for seed bacterization. The seeds were surface-sterilized

with 0.1% $HgCl_2$ [Mercury (II) chloride] for 3-5 min and then washed and rinsed in sterilized distilled water for 3-4 times and dried overnight under a sterile air stream. Cells were grown in TSB for 24 hr at 28-30°C under shaker conditions and were finally centrifuged at 7000 x g for 15 min. The supernatant was discarded and pellet was washed with sterilized distilled water and re-suspended to obtain a population density of 10^7-10^8 CFU/ml. Seeds were allowed to air dry overnight under aseptic conditions Seeds without bacteria served as control (Bhatia et al., 2008). After planting the seeds in pots the pots were randomly placed under fluorescent illumination of 12-14 hr a day length at ambient temperature. Each pot received the same amount of water. The plants were harvested after specific interval of time and data regarding the growth parameters (root length, shoot length, fresh root weight and fresh shoot weight) were recorded.

RESULTS AND DISCUSSIONS

Colonies obtained on various media were selected and identified by standard microbiological procedures.Later those possessing several plant growth promoting characteristics were further identified, andanalysed .The results obtained werethat the rhizosphere isolate M1 R2(Gen-Bank Acc. No.KF-562002)showed 99% similarity with *Pseudomonas fluorescens* gene for 16S rRNA, sequence, Isolate M7R1 (Acc. No KF-562005) a rhizosphere isolate showed 99% similarity with*Pseudomonas aeruginosa* strain P-5 16S ribosomal RNA gene, Bulksoil isolate M6S3 (KF-562003) showed 99% similarity with *Enterobactersakazakii* strain ES2016S (99%).Anotherendophytic isolate M8 ER3 was identified by biochemical testing belonged to *Enterobacter* spp.

In this study conducted 90% of the soil bacteria, 54% of the rhizosphere bacteria and 72% of endorhizosphere bacteria were able to grow in a medium with ACC as a sole source of Nitrogen. DF (minimum salt solution) broth study also showed the presence of growth in the tubes amended with ACC as a sole source of nitrogen.

The resultsof the broth assay showed that *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*and*Enterobacter*were able to utilise ACC as a sole source of Nitrogen when grownin minimal salts medium amended with Ammonium sulphate and ACC.(Table 1).The growth recorded in DF salts medium and in plates ofNFb medium amended with ACC as a sole source source of Nitrogen is shown in (Table 2) and growth in DF broth media is shown in the(Figure 1). *Pseudomonas* and *Enterobacter* species were highly positive.

When grown in a little modified medium the same isolates did not perform well. The positive isolates were further analysed for their plant growth promoting ability by the plate bacterization assay.Highest vigor index was observed in*Pseudomonas* spp.(Table 3).Results of pot assay showed that in sterile soil the percentage increase of root length was from 83-240% whereas in non-sterile soil the root length rangedfrom 43-125%.(Table 4) shows the increase of the selected bacterial isolates.The percentage increase in fresh weight of the plants in sterile soil ranged from 5-71% and in non-sterile soil it was 43-125%. In the pot experiments when the plants were grown in sterile and non-sterile conditions elongation of roots was observed

DISCUSSIONS

PGPR are capable of reducing higher levels of ethylene in plants through the activity of enzyme ACC deaminase. Shaharoona et al. in 2006 observed that inoculation of rhizobacterial strains possessing ACC deaminase significantly increased the root elongation up to 2.4-fold, shoot length up to 2.3-fold, root weight and shoot weight of lentil seedlings as compared to uninoculated control. ACC deaminase production in our study was observed in 72% of the bacterial species. Root length increase over uninoculated control ranged from 83-240% in case sterile soil conditions whereas in non-sterile

conditions increase was from 43-125%. Shoot length increased several folds as compared to uninoculated control.Similar kinds of findings have been reported by other scientists also.(Glick, Penrose and Li 1998;Belimov, Safranova and Minura, 2002; Dodd et al., 2004; Mayak, Tirosh and Glick, 2004; Nadeem et al., 2007; Penrose, Moffat and Glick, 2001; Sergeeva, Shah and Glick, 2005).

A study conducted by Zahir et al. (2008) showed that inoculation with the rhizobacteria containing ACC deaminase caused significant increase in the root length of pea that ranged from 20-80% over the uninoculated control. The shoot length of the pea seedlings was also significantly increased by inoculation with most of the rhizobacteria containing ACC deaminase that ranged from 7-41% over the uninoculated control. Many other researchers have also reported increased resistance to stresses like salt stress (Mayak et al. 2004), flooding stress (Grichko and Glick, 2001), heavy metals (Grichko, Filby and Glick, 2000), drought(Zahir et al., 2008)and pathogen stress (Wang et al., 2000) in response to inoculation with rhizobacteria containing ACC-deaminase. Jing, He and Yang (2007) reviewed recent advances in effect and significance of Rhizobacteria in phytoremediation of heavy metal contaminated soils. Cd in soil induces plant-stress ethylene biosynthesis (Pennasio and Roggero, 1992) and probably contributes to the accumulation of ACC in roots, the PGPR protect the plants against the inhibitory effects of cadmium (Dell'Amico, Cavalca and Andreoni, 2008). ACC deaminase lowers the ethylene production under cadmium stress condition when measured *in vitro* ethylene evolution by wheat seedlings treated with ACC deaminase positive isolates (Govindasamy et al., 2009). Seed inoculations with PGPR in asparagus (*Asparagus officinalis* L.) results in a positive response and enhances plant growth under drought (Liddycoat, Greenberg and Wolyn, 2009).

It is also observed that nodulation and subsequence nitrogen fixation by lentil plants are inhibited by accelerated ethylene concentration in the root zone. PGPR bacteria help overcome these deleterious effects. Thus microorganisms can impart some degree of tolerance to plants towards abiotic stresses like drought, chilling injury, salinity, metal toxicity and high temperature. Bacteria belonging to different genera including *Rhizobium*, *Bacillus*, *Pseudomonas*, *Pantoea*, *Paenibacillus*, *Burkholderia*, *Achromobacter*, *Azospirillum*, *Microbacterium*, *Methylobacteriumvariovorax*, *Enterobacter* etc. have been reported to provide tolerance to host plants under different abiotic stress environment. Alami et al. (2000) reported that *Rhizobium* in association with Sunflower crop through soil aggregation through EPS could help tolerate drought conditions. Sandhya et al. 2009 also suggested that *Pseudomonas putida* P45 could help Sunflower plant tolerate drought conditions (Sandhya et al., 2009a, b). Several PGPR like *Pseudomonas putida*, *Enterobacter cloacae* synthesize ACC deaminase during flooding in tomato crop as reported by Grichko and Glick (2001) which is also reported in our study.Mayak et al. (2004) and Dodd et al. (2005) reported that synthesis of ACC deaminase by *Achromobacterpiechandii*, *Variovoraxparadoxus* tolerate drought in tomato and pea respectively.

Recent reports of several positive attributes of ACC deaminase containing PGPR have also been shown byGrover et al. (2011) in rice and sugarcane.Sarvanakumar and Samiyappan and Chang et al. in 2007 also reported synthesis of ACC deaminase in *Pseudomonas fluorescens* and *Pseudomonas putida*respectively. This study is also the first of its kind in this regionwhich reports that *Pseudomonas* spp. and *Enterobacter* spp. isolated from rhizosphere and associated regions produced ACC deaminase inbroth and plate assayexperiments. Hence thesebacteria also could promote plant growth in early stages so can be used as PGPRand as Bioinoculants to help oleaginous crop plants to survive in stressful environments.

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Table 1: Observation of Growth in Minimal Salts Medium with Ammonium Sulphate and ACC

Isolate No.	O.D 550 nm	Isolate No.	O.D 550nm
M1R2	0.731	M1R2	0.049
M6S3	0.213	M6S3	0.063
M7S1	0.202	M7S1	0.037
M7R1	1.086	M7R1	0.069
M8ER3	0.977	M8ER3	0.04

Table 2: Results Showing Growth in Plates of DF Salts and NFB Medium

Growth in plates in DF salts medium with ACC		Growth in plates with NFB with ACC	
Isolate No.	Inference	Isolate No.	Inference
M1R2	++	M1R2	++
M6S3	+++	M6S3	++
M7S1	+	M7S1	++
M7R1	++	M7R1	++
M8ER3	++	M8ER3	+++

+ scanty growth

++ moderate growth

+++ abundant growth

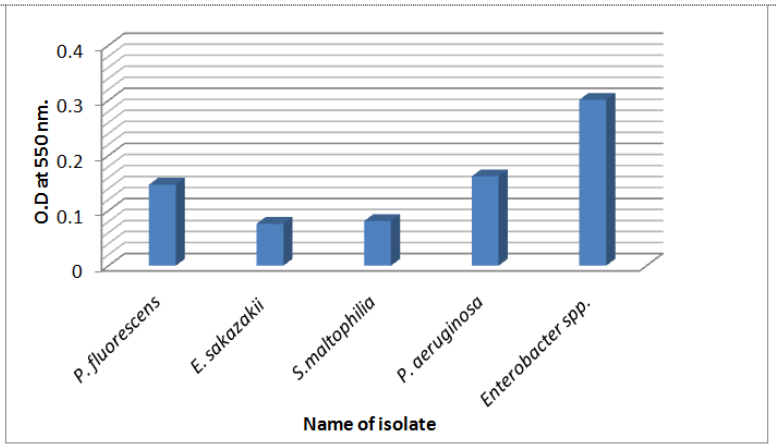

Figure 1: Graphical Representation of Growth in DF Broth Medium

Table 3: Growth Parameters of the Bacterial Isolates in Plate Bacterization Assay

Isolate. No.	Bacterial species	Root length (cm)	Shoot length(cm)	Vigor index	Germination%
	control	5.6	3.1	609	70%
M1R2	*Pseudomonas fluorescens*	9.6	5.2	1480	100%
M7R1	*Pseudomonas aeruginosa*	4.2	5.5	582	60%
M6 S3	*Enterobacter sakazakii*	5.4	6.6	840	70%
M7S1	*Stenotrophomonas maltophilia*	9	5.2	1420	100%
M8 ER3	*Enterobacter spp.*	4.6	2.2	476	70%

Table 4: Results Showing Various Growth Parameters in Pot Assay Experiments

Bacterial Species	Root length (cm)		Shoot length (cm)		Total Fresh Wt. (g)	
	Non sterile soil	Sterile soil	Non sterile soil	Sterile soil	Sterile soil	Non sterile soil
Control	4	3	17	13	0.475	0.592
Pseudomonas fluorescens	6.5	7	17.4	19	0.66	0.65
Pseudomonas aeruginosa	8.5	7	23.2	22	0.81	0.894
Enterobacter sakazakii	5.7	6.4	12	16	0.498	0.256
Stenotrophomonas maltophilia	9	10.2	15.1	18	0.68	0.475
Enterobacter spp.	5.5	6	21	18	0.568	0.648

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